

Electron trap for DNA-bound repair enzymes: A strategy for DNA-mediated signaling

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Despite a low copy number within the cell, base excision repair (BER) enzymes readily detect DNA base lesions and mismatches. These enzymes also contain $[\text{Fe}_4\text{S}_4]$ clusters, yet a redox role for these iron cofactors had been unclear. Here, we provide evidence that BER proteins may use DNA-mediated redox chemistry as part of a signaling mechanism to detect base lesions. By using chemically modified bases, we show electron trapping on DNA in solution with bound BER enzymes by electron paramagnetic resonance (EPR) spectroscopy. We demonstrate electron transfer from two BER proteins, Endonuclease III (EndoIII) and MutY, to modified bases in DNA containing oxidized nitroxyl radical EPR probes. Electron trapping requires that the modified base is coupled to the DNA π -stack, and trapping efficiency is increased when a non-cleavable MutY substrate analogue is located distally to the trap. These results are consistent with DNA binding leading to the activation of the repair proteins toward oxidation. Significantly, these results support a mechanism for DNA repair that involves DNA-mediated charge transport.

base excision repair | DNA charge transport | iron sulfur clusters

Our laboratory has carried out a range of studies to probe and apply DNA-mediated charge transport (CT) chemistry (1). Oxidative damage to DNA from a distance through DNA CT was first demonstrated in an assembly containing a tethered metallointercalator (2). Since that time, photoinduced oxidative damage of the 5'-G of 5'-GG-3' sites from a distance has been observed in assemblies using various pendant oxidants (3–5). Indeed, long-range oxidative DNA damage has been demonstrated over a distance of at least 200 Å (6, 7). Besides the shallow distance dependence in reactions by DNA CT, another characteristic of this chemistry has been its exquisite sensitivity to perturbations in base pair structure. CT through DNA is inhibited by intervening DNA mismatches and bulges as well as by DNA-binding proteins that interfere with base pair stacking (8–12). Recently, studies probing long-range reductive chemistry on DNA also have been investigated both in solution (13–15) and on DNA-modified surfaces (12, 16). As with oxidation chemistry, these reactions show only small variations in rate with distance but are sensitive to perturbations in the intervening base pair stack.

Given the unique characteristics of DNA CT chemistry, we are interested in whether DNA CT might be important physiologically. Based on the exquisite sensitivity of DNA CT to base pair lesions and mismatches, we considered in particular that DNA CT might be advantageous with respect to DNA repair. Base excision repair (BER) enzymes are exceedingly efficient in detecting DNA base lesions and mismatches, yet an understanding of that process has been elusive (17). Interestingly, ubiquitous to these enzymes in homologues from bacteria to man are $[\text{Fe}_4\text{S}_4]^{2+}$ clusters (18). Commonly, $[\text{Fe}_4\text{S}_4]^{2+}$ clusters play redox roles within the cell (19).

MutY and EndoIII are examples of *Escherichia coli* BER glycosylases that contain $[\text{Fe}_4\text{S}_4]^{2+}$ clusters and repair 8-oxo-G:A mismatches and oxidized pyrimidines, respectively (18). The function for the clusters in these enzymes was unclear, because

redox activity in the physiological range of potentials was not found (20). Although these enzymes have different substrates, they are similar structurally (21–24). The $[\text{Fe}_4\text{S}_4]^{2+}$ clusters are well separated from the enzyme active site and do not appear to participate in the glycosylase reaction, yet they are essential for overall repair activity. Crystal structures in the absence and presence of DNA, moreover, show that binding to DNA significantly changes the environment of the cluster, taking the cluster loop from a relatively exposed polar environment to a more hydrophobic one near the DNA groove.

By using DNA-modified electrodes, we have found that, bound to DNA, both MutY and EndoIII are indeed redox-active at physiological potentials (25, 26). On DNA-modified electrodes, the proteins display midpoint potentials of 59 and 90 mV vs. NHE, potentials characteristic of high-potential iron proteins (27). EPR experiments at 10K with these BER enzymes also directly show activation of the clusters toward oxidation upon DNA binding (26). Thus, although resistant to oxidation and reduction in solution, DNA binding appears to shift the $[\text{Fe}_4\text{S}_4]^{3+/2+}$ redox potential, activating the protein toward oxidation. Importantly, we also have seen that guanine radical can promote oxidation of the $[\text{Fe}_4\text{S}_4]^{2+}$ cluster of MutY through DNA-mediated CT so as potentially to activate repair by MutY under conditions of oxidative stress (28).

Fig. 1 illustrates our model for how these BER enzymes might use DNA CT in locating DNA lesions and mismatches. The protein, not bound to DNA, is redox inactive. Binding to DNA, however, favors oxidation of the protein, with DNA-mediated electron transfer to a second BER enzyme bound at a distal position; reduction of this alternate protein then favors its dissociation. Given the sensitivity of DNA CT to intervening base lesions, this reaction can proceed only if the DNA base pair π -stack is intact. When the path between DNA-bound proteins is interrupted by a base lesion, DNA-mediated CT reaction is inhibited, and, on a slower time scale, the protein can move processively (29, 30) to the site and fix it. Hence, DNA CT provides a rapid strategy to redistribute DNA repair proteins onto regions of the genome containing lesions.

In this proposal, then, DNA-mediated CT provides the key element in signaling among the repair proteins to scan the genome for lesions. It therefore becomes important to establish directly this key step. Here, we provide evidence in support of this step by demonstrating trapping of an electron on DNA associated with protein binding.

Results

EPR Experiments with EndoIII and MutY. Fig. 2 illustrates the experimental strategy for electron trapping on the DNA duplex. DNA assemblies were synthesized containing a uracil base modified with an alkyne linked to a nitroxyl radical spin label (31). This nitroxyl radical, incorporated in DNA, is EPR-active

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Abbreviations: BER, base excision repair; CT, charge transport.

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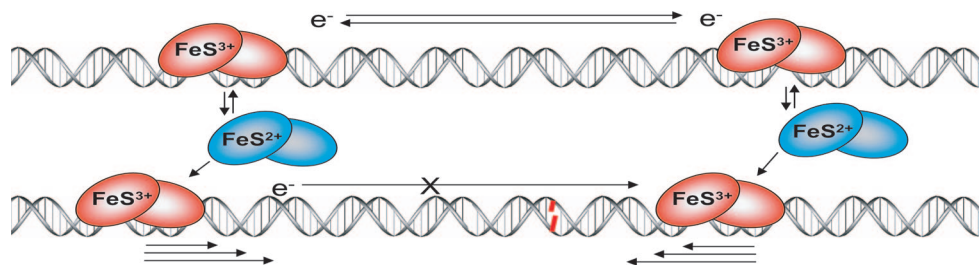


Fig. 1. Proposed model for long-range DNA signaling between BER enzymes using DNA-mediated CT to detect base lesions. In the absence of DNA, the $[\text{Fe}_4\text{S}_4]^{2+}$ clusters in the BER enzymes are robust to oxidation. Binding to DNA activates the BER enzymes to oxidation, facilitating DNA-mediated CT to an alternate protein bound at a distal site. Reduction of the distally bound protein then promotes its dissociation. Binding of a protein near a base lesion, however, precludes DNA-mediated CT; hence, the protein remains localized in the vicinity of the lesion and can diffuse processively to the site to excise the base. DNA CT therefore promotes a redistribution of the repair enzymes near sites in need of repair.

in solution at ambient temperatures. Oxidation of the nitroxyl radical to the EPR-silent diamagnetic *N*-oxo-ammonium ion ($\text{R}-\text{N}=\text{O}^+$) can be attained with a mild oxidant, IrCl_6^{2-} , that does not promote damage to the natural bases (32). The DNA probe, once oxidized, provides the electron trap upon reaction with repair enzymes. Because the reduction potential of $\text{R}-\text{N}=\text{O}^+$ bound to the uridine base is ≈ 1 V vs. NHE (data not shown) and the $[\text{Fe}_4\text{S}_4]^{3+/2+}$ midpoint potential is 0.1 V vs. NHE for the protein bound to DNA (26), electron transfer from the

$[\text{Fe}_4\text{S}_4]$ cluster of the protein to the *N*-oxo-ammonium should be favorable. Thus, the repair protein, once bound to DNA, is expected to reduce the EPR-silent *N*-oxo-ammonium ion back to the EPR-active nitroxyl radical. Note the potentials for reduction of the BER enzymes of ≈ 60 and 90 mV vs. NHE are insufficient to reduce the intervening bases (33).

Fig. 3 shows the results. EPR spectra of DNA samples consisting of a fully matched 36-mer duplex were acquired at ambient temperature before and after addition of IrCl_6^{2-} and subsequent to protein binding. With the addition of Ir(IV), a substantial decrease in the EPR signal is observed, indicating efficient oxidation of the spin label from the nitroxyl radical to the EPR silent *N*-oxo-ammonium ion. The nitroxyl radical signal does not reappear for several hours in the absence of protein, indicating the stability of the *N*-oxo-ammonium ion (data not shown). Significantly, however, addition of either MutY or EndoIII results in the significant regeneration of the EPR signal.

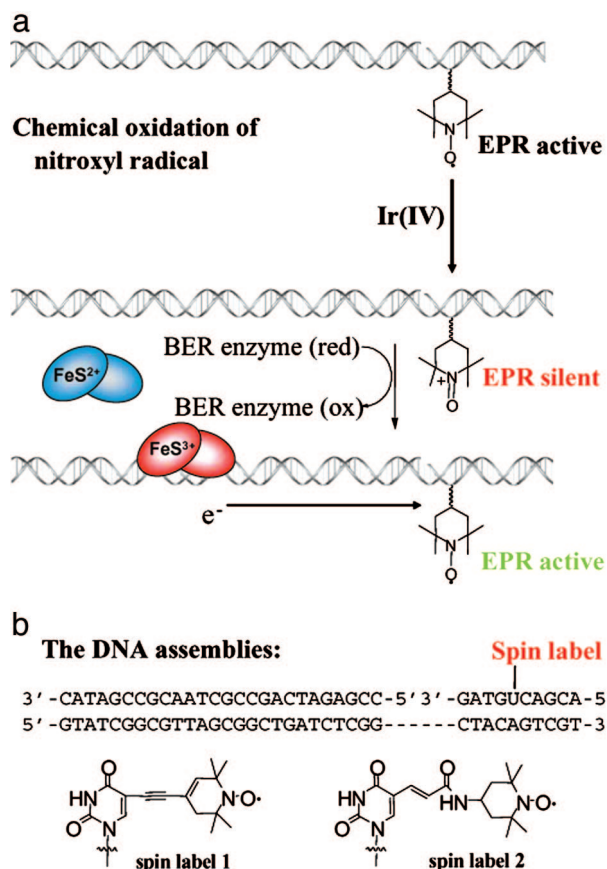


Fig. 2. Electron trapping on the DNA duplex. (a) The methodology used. The stable nitroxyl radical is covalently attached to a uridine base on the DNA duplex. This organic radical may be readily monitored by EPR at ambient temperature. Mild oxidation with Ir(IV) of the nitroxyl radical generates the diamagnetic *N*-oxo-ammonium species. Upon protein binding, electron trapping on the DNA then can be monitored by the reappearance of the EPR signal of the nitroxyl radical. (b) The DNA assemblies and modified bases used.

EPR Experiments with a Noncleavable Substrate Analogue of MutY.

Two routes were explored to determine whether the protein-dependent reduction of the modified base occurs in a DNA-mediated reaction as opposed to direct binding of the protein to the modified base. MutY was previously shown to bind preferentially to the 7-deaza-adenine:guanine (ZG) base pair but not to remove the modified adenine (34). We find here that addition of MutY to the assembly containing the ZG base pair separated by 19 base pairs from the electron trap results in a significantly greater regeneration of the EPR signal in comparison with an identical duplex containing instead a CG base pair (Fig. 4). With duplex DNA lacking the ZG base pair, 47% regeneration of the nitroxyl radical signal is seen (relative to the nitroxyl radical signal on the DNA before oxidation), whereas with the DNA assembly containing the ZG base pair, 70% signal regeneration is consistently found. The increase in signal is attributed to the higher binding affinity for this site. Thus, the result supports protein binding to the ZG base pair. Because the ZG base pair is well separated from the *N*-oxo-ammonium ion, this result points to protein reduction of the modified base necessarily arising from a distance.

EPR Experiments with a Poorly Coupled Spin-Labeled Probe.

Another strategy used to test whether CT is DNA-mediated takes advantage of an alternate electron trap that is not well coupled into the base pair stack. As is evident in Fig. 5, with a modified base containing the nitroxyl radical in a saturated ring (spin label 2), addition of Ir(IV) results also in complete oxidation of the nitroxyl radical. Significantly, however, the addition of EndoIII leads only to a small regeneration of the signal. This result might be expected with the limited binding of protein directly neighboring the probe. Thus, the protein clearly does not bind preferentially to the modified base but instead binds to many

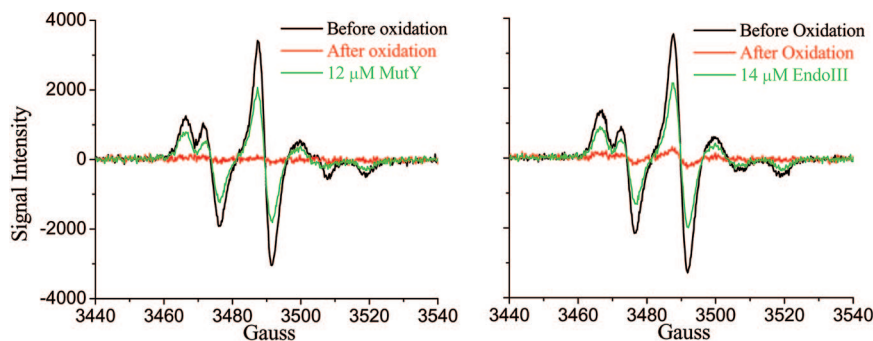


Fig. 3. EPR spectra before and after addition of MutY (Left) and EndoIII (Right) are shown for DNA duplexes (7 μM) initially treated with IrCl_6^{2-} (60 μM). Spectra shown are before oxidation (black), after oxidation before protein addition (red), and then after protein addition (green). Protein concentrations are 12 μM for MutY and 14 μM for EndoIII.

sites on the DNA duplex. With the probe that is electronically coupled into the base pair stack through a conjugated system with olefin and alkyne linkages (spin label 1), CT can occur from a distance through the DNA. However, long-range CT cannot proceed efficiently to the *N*-oxo-ammonium ion through the saturated ring (spin label 2). These data therefore further support that protein-dependent reduction of the well coupled modified base is DNA-mediated.

Discussion

***N*-Oxo-Ammonium Ion as an Electron Trap on DNA.** These experiments illustrate the utility of a nucleic acid base modified with *N*-oxo-ammonium ion as a sensitive probe of reductive chemistry on DNA. The experiment is carried out at ambient temperatures so that enzyme decomposition, a consideration in low-temperature EPR experiments, is not an issue. The experiment is highly reproducible and remarkably sensitive. This strategy may be used generally to test for DNA-mediated reductive chemistry by different DNA-binding proteins containing redox cofactors as well as to test for long-range CT chemistry promoted by synthetic reductants.

EndoIII and MutY Inject Electrons into the DNA Base Pair Stack. The base modified with *N*-oxo-ammonium ion and coupled into the base pair stack clearly provides an effective electron trap on the DNA for these repair proteins. These data support the reduction of the modified base in the DNA assemblies by DNA-bound MutY and EndoIII. Importantly, DNA CT occurs at potentials that are insufficient to promote reaction of the natural bases.

Is the CT reaction DNA-mediated? Two experiments are consistent with reduction of the modified base probe from a distance. First, providing an alternate preferred site on the DNA for the repair protein leads to an increased yield of reduction, rather than a decrease as might be expected with direct reduction of the modified base by protein bound locally. Additionally, uncoupling the *N*-oxo-ammonium ion from the DNA base pair stack through a saturated ring leads to diminished reduction of the modified base; poor coupling into the base stack inhibits long range CT. Without covalent attachment of the protein to DNA, CT from a distance cannot be established definitively. Nonetheless, these observations provide strong support for electron trapping in a DNA-mediated reaction.

DNA-Mediated Signaling in DNA Repair. It is apparent that binding to DNA promotes redox chemistry by these BER enzymes and

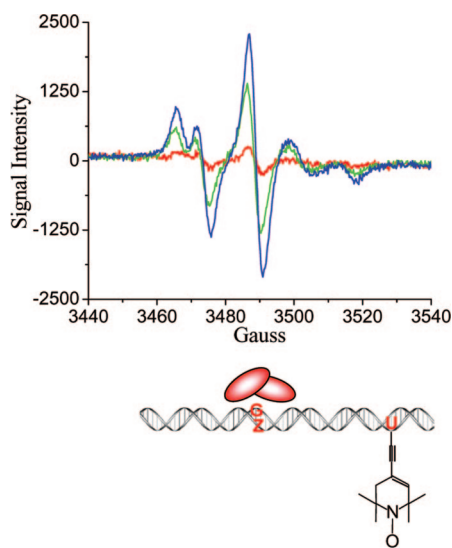


Fig. 4. EPR spectra of a DNA duplex (6.6 μM) containing the nitroxyl radical-modified base separated by 19 base pairs from a GZ base pair after (red) treatment with IrCl_6^{2-} (60 μM) followed by the addition of 6.2 μM MutY (blue). An increase in the signal intensity is observed in comparison with an identical duplex lacking the GZ base pair (green).

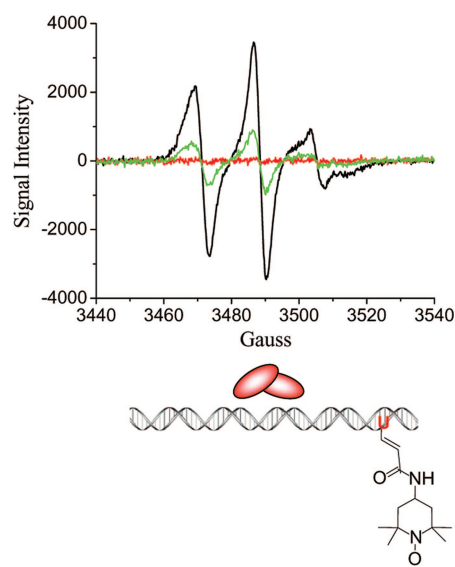


Fig. 5. EPR spectra of a DNA duplex (7 μM) containing a poorly conjugated spin label (spin label 2, black) initially treated with 60 μM IrCl_6^{2-} (red) followed by the addition of 14 μM EndoIII (green).

through a DNA-mediated reaction. These data therefore provide direct support for our model of DNA-mediated signaling by BER proteins (Fig. 1). Electron injection into the base pair stack is associated with DNA binding by the BER proteins and does provide a means to rapidly detect lesions and mismatches, because such perturbations in the π -stack inhibit DNA CT. If no stacking perturbations are detected, CT among proteins can ensue, leading to their redistribution onto alternate sites in the genome. If stacking disruptions inhibit CT among the proteins, then no relocation of the proteins occurs, and, on a slower timescale, the protein locally bound can move processively to the site and begin repair.

Consistent with our model, then, DNA-mediated signaling between DNA-bound BER enzymes is feasible. Because DNA CT is exquisitely sensitive to base lesions, this DNA-dependent redox chemistry provides an attractive and reasonable strategy for the detection of base lesions, the first step in DNA repair.

Materials and Methods

Materials. All buffers were freshly prepared and filtered before use. All reagents for DNA synthesis were purchased from Glen Research (Sterling, VA). Chemical reagents were purchased from Aldrich and used without any further purification. Solvents (pyridine and tetrahydrofuran) were purchased from Fluka (dried over molecular sieves) and used as obtained.

Synthesis of Oligonucleotides. Synthesis of spin label **1** was carried out according to literature procedures with a slight modification: The dimethoxytrityl-protected 5-iodo-uridine was Pd coupled (35) to the acetylene-modified spin-labeled 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) derivative in tetrahydrofuran. Using a solvent without amines improved the yields significantly (36). The product of this reaction then was activated for DNA synthesis after incorporating the phosphoramidite onto the 3'-OH end of the sugar. The synthesis of a 10-mer DNA strand that included spin label **1** was accomplished by standard automated DNA synthesis on an Applied Biosystems 394 DNA synthesizer using dichloroacetic acid (instead of trichloroacetic acid) as the deblocking reagent. The second spin-labeled uridine probe (spin label **2**) was obtained by synthesizing, using standard automated methods, a 10-mer DNA with a terminal carboxyl group on a uridine base (Carboxy dT, Glen Research). After mild deprotection from the resin (0.4 M NaOH in 4:1 MeOH:H₂O; ambient temperatures/overnight) the DNA strand was purified by HPLC. Next, the 5' *O*-DMT protected 10-mer DNA (150 nmol) was coupled in solution at ambient temperatures (24 h) to 4-amino-TEMPO (150 μ mol) using 1-(3-dimethylamino-propyl)-3-ethyl-carbodiimide hydrochloride (15 μ mol) and sulfo-*N*-hydroxysuccinimide (15 μ mol) as coupling reagents in a 0.1 M MES/0.5 M NaCl buffer (pH 6, 80 μ l), followed by EtOH (400 μ l) precipitation.

All DNA strands were purified by HPLC on a C18 reverse-phase column and characterized by MALDI-TOF mass spectrometry. DNA sequences include the following: (i) a 10-mer spin-labeled DNA 3'-GATGU**CAGCA*-5' (where U* = spin labels **1** or **2**); (ii) 26-mer DNAs 3'-CATAGCCGCAAX¹CGC-CGACTAGAGCC-5' (where X¹ = T = sequence **A** and X¹ =

G = sequence **B**); and (iii) 36-mer DNAs 5'-GTATCGG-CGTTX²GC-GGCTGATCTCGGCTACAGTCGT-3' (where X² = A for a fully matched duplex when annealed to sequence **A** and X² = Z for a fully matched duplex with a GZ base pair when annealed to sequence **B**).

DNA Annealing. DNA annealing was carried out initially by heating a solution containing the 26:36-mer strands in 10 mM NaPi/50 mM NaCl (pH 7.5) at a 1:1 ratio to 90°C, followed by slow cooling over 90 min to room temperature. Next, the 10-mer spin-labeled DNA (**1** or **2** in a 0.9:1 ratio to the 26- to 36-mer duplex) was added to the preassembled 26- to 36-mer duplex by heating the solution to 45°C for 2 min and allowing it to cool to ambient temperature. Duplex formation with the 10-mer spin-labeled DNA was verified by EPR spectroscopy. It should be noted that the addition of a glycerol-containing buffer to DNA samples post treated with Ir(IV) resulted in a signal regeneration of the nitroxyl radical (data not shown); however, this process was much slower (hours) in comparison with that with the different proteins examined.

Protein Preparation. MutY (37) and EndoIII (38, 39) were prepared as described.

EPR Spectroscopy. X-band EPR spectra were obtained on a EMX spectrometer (Bruker Billerica, MA) equipped with a rectangular cavity working in the TE₁₀₂ mode. A quartz flat cell (100 μ l) was used in all room temperature experiments. A frequency counter built into the microwave bridge provided accurate frequency values. DNA samples consisted of 26–36 preassembled duplexes (8 μ M) and 10-mer spin-labeled DNA (7.2 μ M) in 75 μ l of buffer (10 mM NaPi/29 mM NaCl/2.6 mM MgCl₂, pH 6.2). Chemical oxidation of the spin-labeled probe then was accomplished by addition of 2 μ l of K₂[IrCl₆] (2.5 mM in 10 mM NaPi/50 mM NaCl, pH 7.5), as determined by a significant attenuation in the EPR signal. After this oxidation, protein (EndoIII or MutY) was added to the sample, and the spectrum was immediately recorded (typically after 2 min). EPR spectra also were recorded at longer times (4–12 min) until the EPR signal intensity was constant. EPR parameters for ambient temperature measurements were as follows: microwave power = 20 mW, receiver gain = 1×10^4 , and modulation amplitude = 4G.

Electrochemistry. The oxidation potential of the spin-labeled deoxyuridine compound was determined by cyclic voltammetry using a CV-50W instrument (Bioanalytical Systems, West Lafayette, IN). Measurements used a glassy carbon working electrode and an Ag/AgCl reference electrode. The compound was dissolved in dry acetonitrile containing 0.1 M tetrabutylammonium hexafluorophosphate as the supporting electrolyte. A scan rate of 0.1 V/s was used in these measurements.

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